Part3：Animal experiments

To simulate how our system works in real world condition, we transformed our devices into B. *lougum* and use these engineered Bifidobacterium to treat tumor bearing mice.

First, we transformed our devices (BBa_K1932005, BBa_K1932006, BBa_K1932007) and pUC-18 vector into E. *coli* BL21 competent cells (Fig.3.1). BBa_K1932005 is a device which contains the sequence of TAT-apoptin. BBa_K1932006 is a device which contains the sequence of Sec2-TAT-apoptin and BBa_K1932007 is a device which contains the sequence of Tmp1-TAT-apoptin.

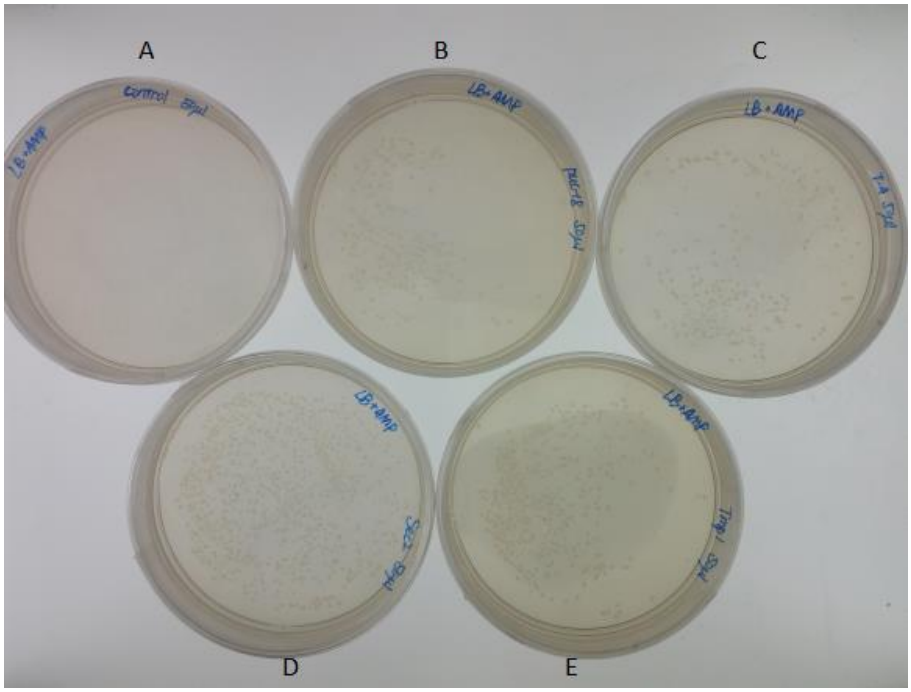

Fig.3.1.Transformation of E. *coli* BL21: control (A), pUC-18 vector (B), pUC-18-TAT-apoptin (C), pUC-18-Sec2-TAT-apoptin (D), pUC-18-Tmp1-TAT-apoptin (E).

Then we transformed two of our devices, BBa_K1932006 and BBa_K1932007, into Bifidobacterium, by electro-transformation assay (Fig.3.2). BBa_K1932006 is a device which contains the sequence of Sec2-TAT-apoptin and BBa_K1932007 is a device which contains the sequence of Tmp1-TAT-apoptin. We used these two devices because each of they contains a signal peptide gene and a TAT trans-membrane domain gene, with which apoptin can be secreted by Bifidobacterium and be transducted into tumor cells. Next, we use a Lyophilizer to lyophilize the solution of these engineered Bifidobacterium in vacuum environment under -50℃. After that we planned to use these lyophilized powder to treat tumor bearing nude mice. So we calculated the CFU (Colony Forming Unit) of our Bifidobacterium lyophilized powder through plate counting (Fig.3.3).

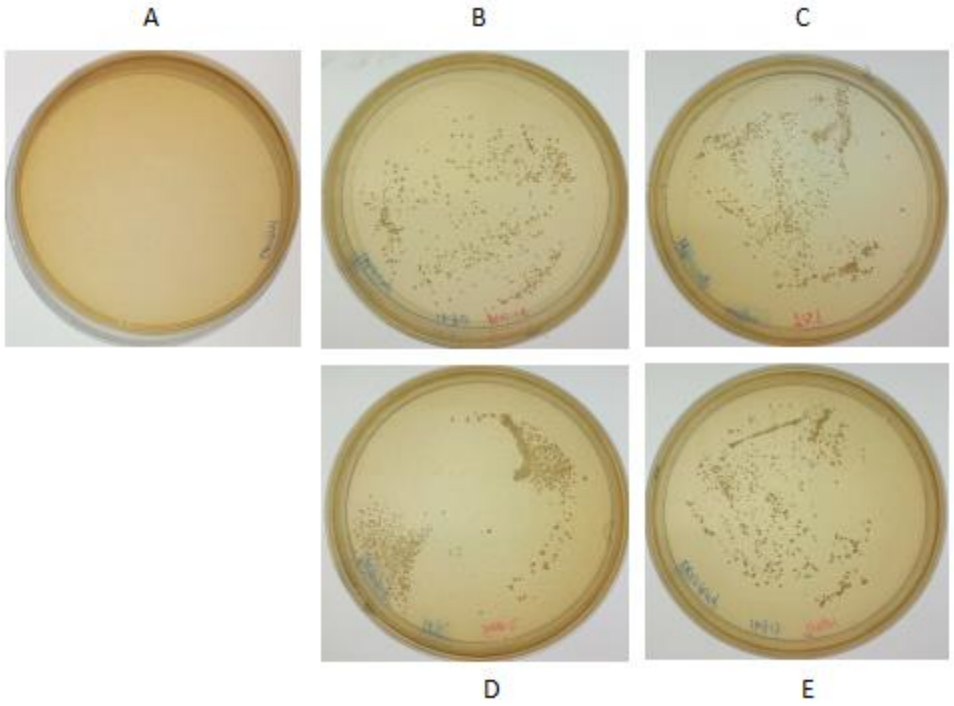

Fig.3.2.Electro-transformation of Bifidobacterium (1250V, 200Ω, 25μf, 1.0mm, 5ms), control (A), pUC-18 vector (B), pUC-18-TAT-apoptin (C), pUC-18-Sec2-TAT-apoptin (D), pUC-18-Tmp1-TAT-apoptin (E).

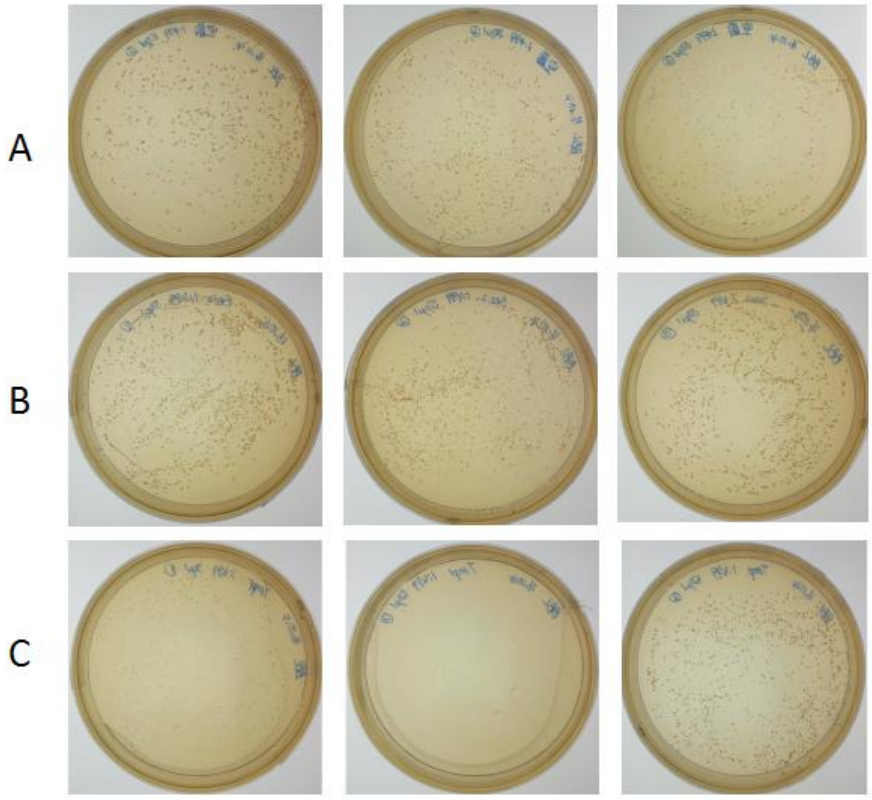

Fig.3.3.The lyophilized powder was diluted with 1 X PBS buffer, and the CFU is calculated by plate counting method, B. *longum* (A), B. *longum* with BBa_K1932006 (pUC-18-Sec2-TAT-apoptin) plasmid (B) B. *longum* with BBa_K1932007 (pUC-18-Tmp1-TAT-apoptin) plasmid (C).

At the same time, we designed the process of constructing a model of liver cancer. Out of the concern

of students' safety, undergraduate students are not allowed to enter laboratory animal environment without license. So we outsource these works to JRDUN Biotechnology. They followed our protocol to construct a model of liver cancer using SMMC-7721 cells (human liver cancer cell line). Ten days after they injected 40 nude mice with 100ul SMMC-7721 cell (2×10^7/ml) to axillae, the liver cancer model was constructed. But only 39 tumor bearing nude mice were survived, so we asked them to choose 35 nude mice which have the tumors of similar volume. Then these mice were treated by in situ injection method.

Then these tumor bearing nude mice were divided into five groups randomly (seven for each):

1. NC group, negative control group, injected 50ul 1X PBS buffer in 10d, 15d, and 20d.
2. PC group, positive control group, injected 50ul doxorubicin hydrochloride at a dosage of 4.3769mg/kg in 10d, 15d, and 20d.
3. BF group, Bifidobacterium control group, injected 50ul B. *longum* at a dosage of $2.5\times10^{\wedge}7$ CFU/kg in 10d, 15d, and 20d.
4. BFS group, Bifidobacterium-Sec2 group, injected 50ul injected B. *longum* transformed with BBa_K1932006 at a dosage of $2.5\times10^{\wedge}7$ CFU/kg in 10d, 15d, and 20d.
5. BFT group, Bifidobacterium-Tmp1 group, injected 50ul injected B. *longum* transformed with BBa_K1932007 at a dosage of $2.5\times10^{\wedge}7$ CFU/kg in 10d, 15d, and 20d.

We used the results of plate counting method to dilute the lyophilized powder with 1X PBS buffer, adjusting the concentration of the solution to $1\times10^{\wedge}7$ CFU/ml. To simulate the process of our engineered Bifidobacterium killing tumor cells in vivo, we inject these solutions into tumor-bearing nude mice (Fig.3.4 and Fig.3.5). The volumes of the tumors were measured every three days (Fig.3.6, Tab.1), and tumor mass were weighed at the last day (Fig.3.7, Tab.2).

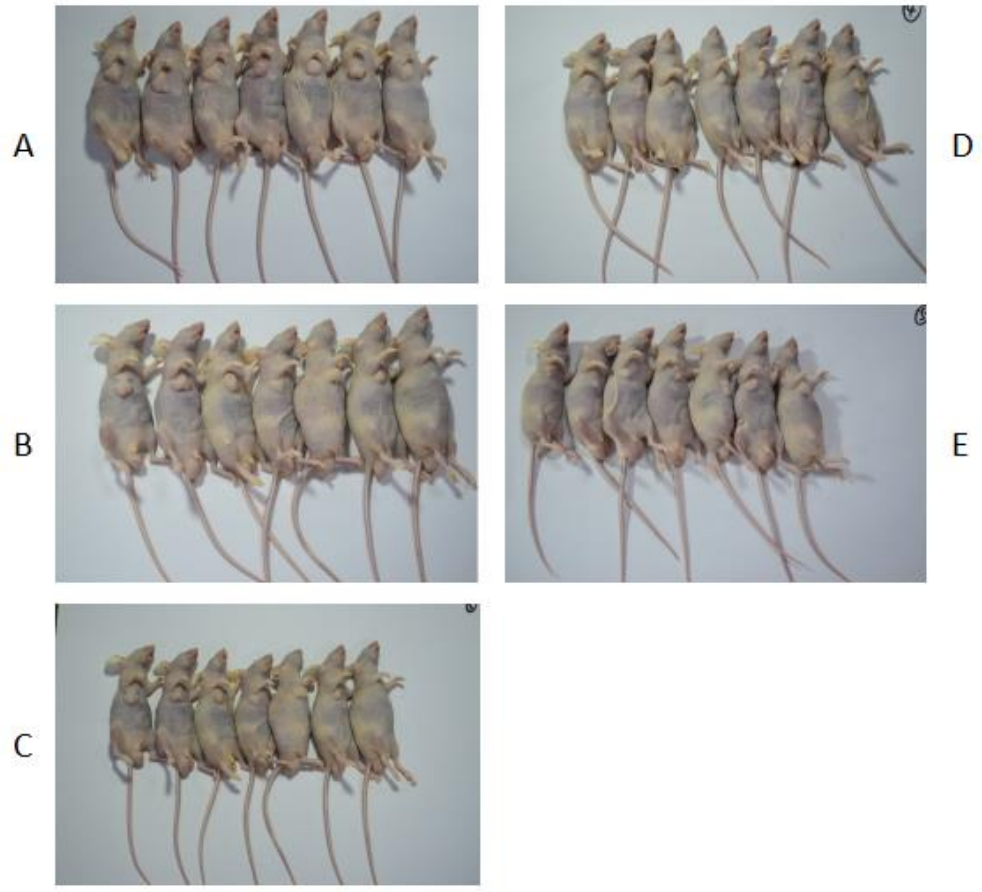

Fig.3.4.Tumor bearing nude mice were injected with the solution of lyophilized powder. (A) Mice in negative control group were injected with 1X PBS buffer. (B) Mice in positive control group were injected with doxorubicin hydrochloride at a dosage of 4.3769mg/kg. (C) Mice in BF group were injected with B. *longum* at a dosage of $2.5\times10^{\wedge7}$ CFU/kg. (D) Mice in BFS group were injected with B. *longum* transformed with BBa_K1932006 at a dosage of $2.5\times10^{\wedge7}$ CFU/kg. (E) Mice in BFT group were injected with B. *longum* transformed with BBa_K1932007 at a dosage of $2.5\times10^{\wedge7}$ CFU/kg.

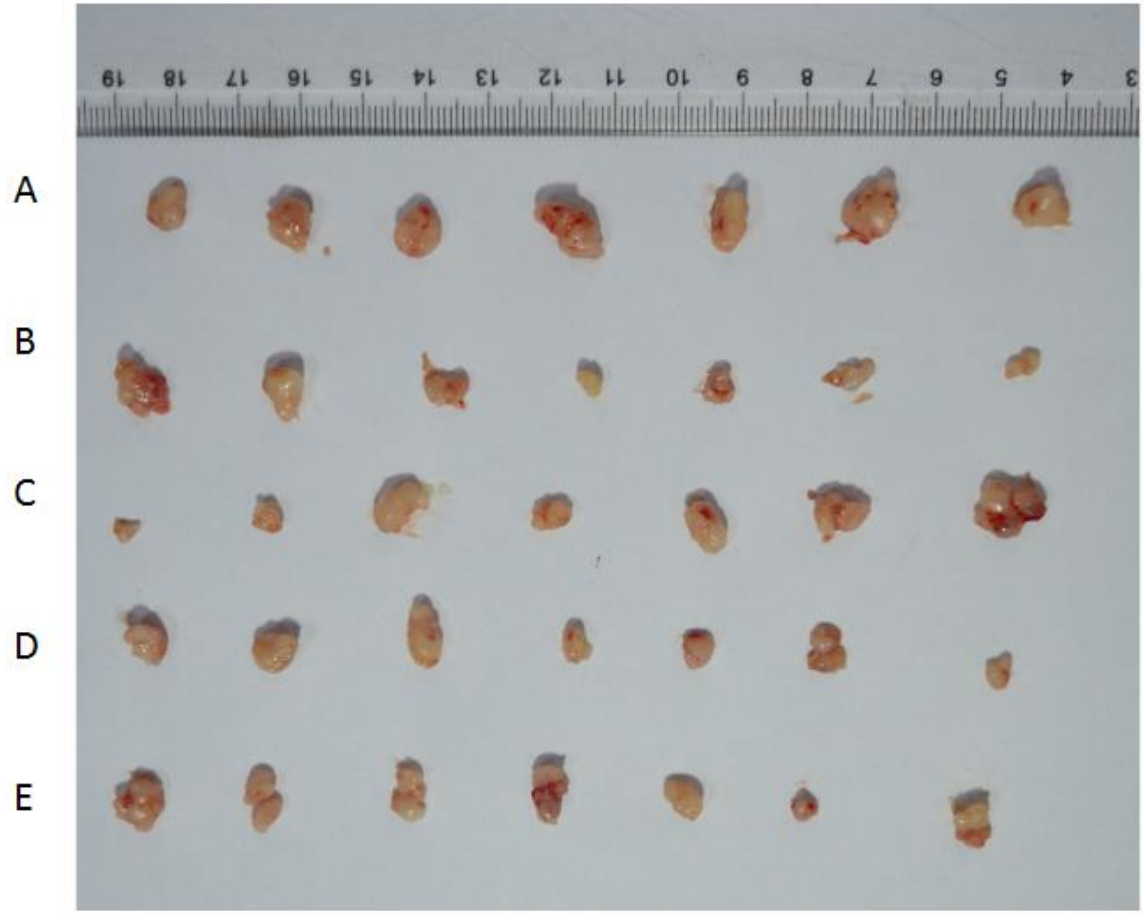

Fig.3.5. Mice were sacrificed by cervical dislocation in 21 days and the tumor mass was photographed. (A) Mice in negative control group were injected with 1X PBS buffer. (B) Mice in positive control group were injected with doxorubicin hydrochloride at a dosage of 4.3769mg/kg. (C) Mice in BF group were injected with B. *longum* at a dosage of $2.5\times10^{\wedge7}$ CFU/kg. (D) Mice in BFS group were injected with B. *longum* transformed with BBa_K1932006 at a dosage of $2.5\times10^{\wedge7}$ CFU/kg. (E) Mice in BFT group were injected with B. *longum* transformed with BBa_K1932007 at a dosage of $2.5\times10^{\wedge7}$ CFU/kg.

A

NC	1		2		3		4		5		6		7	
Days	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)
9	1.59	2.38	1.66	1.86	1.74	2.01	1.94	2.47	1.75	1.82	1.53	1.86	1.33	1.42
12	3.67	3.78	3.87	3.96	3.12	3.56	3.02	4.06	3.31	3.45	3.32	3.76	2.5	2.57
15	5.15	5.42	5.65	5.78	5.11	5.86	4.56	6.32	5.23	5.36	4.56	5.44	3.78	3.89
18	6.93	7.51	6.99	7.66	6.72	7.71	6.02	8.87	6.85	6.9	5.5	7.03	4.9	5.15
21	8.58	9.76	9.07	10.57	8.21	10.56	7.42	12.19	8.12	8.36	6.71	9.96	6.53	6.6

B

PC	1		2		3		4		5		6		7	
Days	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)
9	1.56	1.83	1.65	1.78	1.84	2.01	1.87	2.1	1.71	2.01	1.69	1.89	1.84	1.91
12	2.71	3.65	2.89	3.02	2.83	3.11	2.91	3.03	3.12	3.28	3.15	3.34	3.18	4.09
15	3.34	4.55	3.95	4.11	3.56	4.23	3.78	3.99	4.07	4.89	4.48	5.51	4.91	5.89
18	4.21	5.12	4.77	5.34	4.37	5.38	4.51	4.86	5.11	6.46	6.08	7.32	6.01	7.53
21	4.91	6.53	5.59	6.44	5.33	6.41	5.61	5.93	6.26	8.23	7.47	9.05	7.08	9.39

C

BF	1		2		3		4		5		6		7	
Days	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)
9	1.87	1.92	1.67	2.12	1.77	1.88	1.81	2.02	1.93	1.98	1.57	1.76	1.89	2.05
12	3.98	4.77	3.12	4.56	3.56	4.6	3.28	3.78	3.21	4.67	2.48	2.65	2.48	2.65
15	5.71	6.98	4.53	6.38	6.17	6.3	3.98	5.06	4.47	6.47	3.16	3.54	3.07	3.37
18	7.32	9.01	5.89	8.32	6.3	7.79	4.89	6.21	5.69	8.49	3.79	4.46	3.98	4.09
21	9.74	11.18	7.02	10.43	6.3	9.88	5.87	7.33	6.98	10.55	4.37	5.33	4.83	5.05

D

BFS	1		2		3		4		5		6		7	
Days	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)
9	2.01	2.03	1.71	1.85	1.76	1.78	1.74	1.96	1.8	1.97	1.86	1.89	1.78	2.01
12	2.49	3.21	2.38	3.33	3.12	3.31	3.02	3.34	3.46	3.99	3.28	4.05	3.15	3.2
15	3.12	3.99	2.91	4.28	3.96	4.12	3.81	4.13	4.38	5.91	4.39	5.48	4.23	4.32
18	3.74	5.02	3.56	5.19	4.51	4.77	4.84	5.25	5.41	7.83	5.5	6.98	5.39	5.75
21	4.49	6.07	4.27	6.22	5.34	5.46	5.96	6.74	6.46	9.93	6.79	8.65	7.15	7.41

E

BFT	1		2		3		4		5		6		7	
Days	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)	SD(mm)	LD(mm)
9	1.84	1.98	1.69	1.72	1.87	1.98	1.89	2.03	1.74	2.03	1.79	1.91	1.89	2.07
12	3.32	3.45	2.89	3.04	3.18	3.68	3.08	3.74	3.17	4.18	3.67	4.07	4.32	4.34
15	4.51	4.67	3.48	3.56	3.89	4.85	3.55	4.98	3.78	5.48	4.48	5.32	5.47	5.51
18	5.69	5.89	3.96	4.01	4.53	6.19	4.1	6.46	4.65	7.02	5.09	6.9	6.78	6.98
21	6.84	6.98	4.42	4.59	5.36	7.65	4.63	8.07	5.51	9.63	5.76	8.72	8.36	8.41

Tab.1.The volumes of the tumors were measured every three days (Volume=1/2SD^2*LD, SD: short diameter, LD: long diameter). (A) NC group, (B) PC group, (C) BF group, (D) BFS group, (E) BFT group.

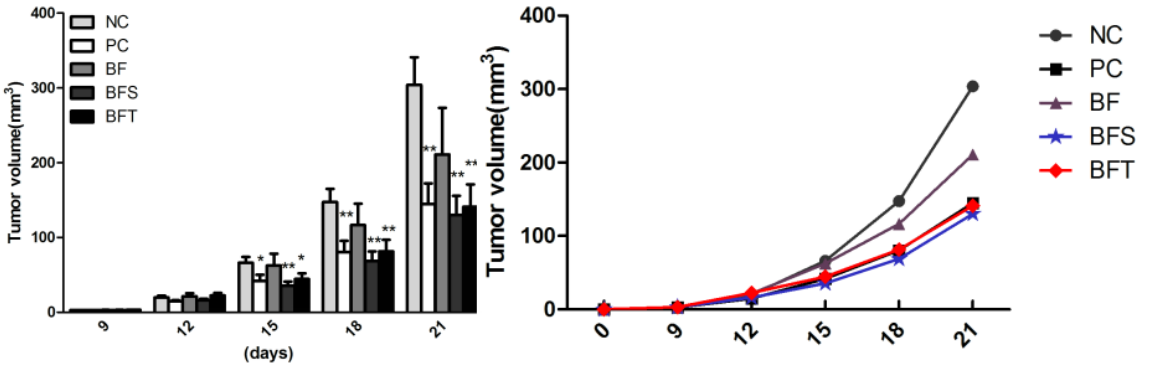

Fig.3.6.The volume of the tumor was measured and recorded every 3 days.

	weight（g）						
Groups	1	2	3	4	5	6	7
NC	0.1387	0.2041	0.1721	0.2801	0.1565	0.1780	0.1077
PC	0.0301	0.0399	0.0327	0.0409	0.0725	0.1619	0.1478
BF	0.2505	0.1546	0.1441	0.0619	0.1697	0.0527	0.0186
BFS	0.0247	0.0546	0.0534	0.0499	0.1483	0.1477	0.1108
BFT	0.1053	0.0212	0.0772	0.1149	0.0886	0.1065	0.1634

Tab.2. The mice were killed by cervical dislocation in 21d and the tumors were weighed.

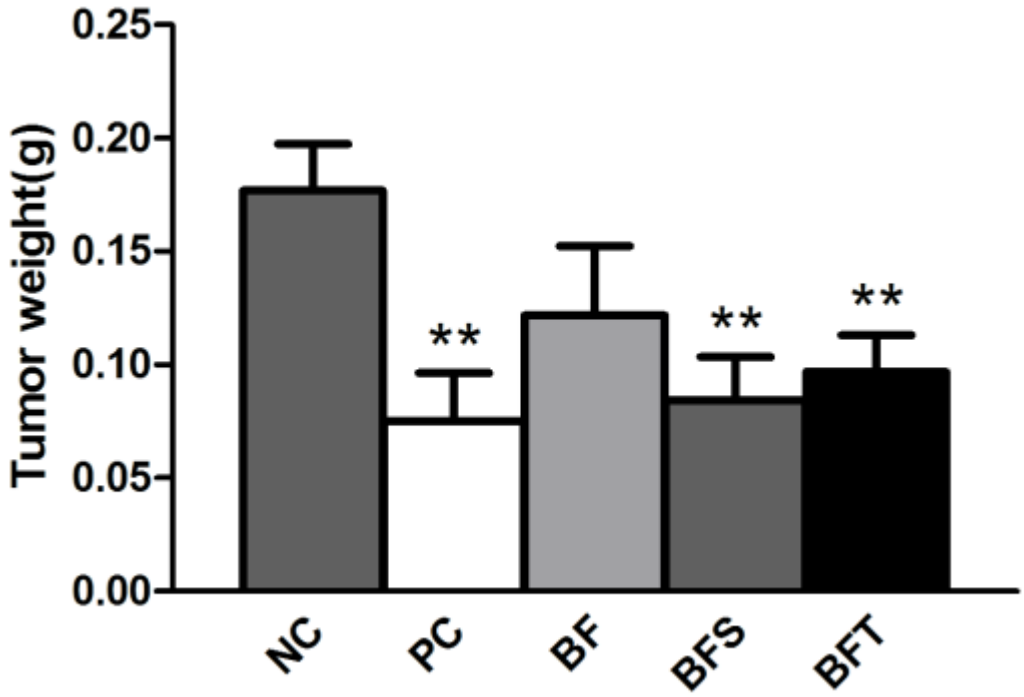

Fig.3.7.The mice were killed by cervical dislocation in 21d and the tumors were weighed.

Compared with NC and BF groups, the tumor growth was retarded in BFS group and BFT group. There was no significant difference on tumor growth between BF group and NC group, indicating *B. longum* alone did not have a significant inhibitory effect on tumor growth.

These results demonstrated that our engineered Bifidobacterium which expresses Sec2-TAT-apoptin protein or Tmp1-TAT-apoptin protein have an inhibiting effect on the growth of solid tumors.